

12/PRTS

514 Rec'd PCT/PTO 31 JAN 2000

Novel composition and method for the detection of diseases associated with amyloid-like fibril or protein aggregate formation

The present invention relates to novel compositions useful for elucidating the onset or progress of diseases of preferably neuronal origin associated with the formation of amyloid-like fibrils or protein aggregates. Further, the present invention relates to methods for monitoring said formation as well as to methods for identifying inhibitors of said formation. Additionally, the invention relates to inhibitors identified by the method of the invention as well as to pharmaceutical compositions comprising said inhibitors.

A variety of diseases, both in humans and animals, is characterized by the pathogenic formation of amyloid-like fibrils or protein aggregates in neuronal tissues. A well-known and typical example of such diseases is Alzheimer's disease (AD). AD is characterized by the formation of neurofibrillar tangles and β -amyloid fibrils in the brain of AD patients. Similarly, scrapie is associated with the occurrence of scrapie-associated fibrils in brain tissue.

Another class of these diseases is characterized by an expansion of CAG repeats in certain genes. The affected proteins display a corresponding polyglutamine expansion. Said diseases are further characterized by a late onset in life and a dominant pathway of inheritance.

A typical representative of this class of diseases is Huntington's disease. Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder characterized by personality changes, motor impairment and subcortical dementia (Harper, 1991). It is associated with a selective neuronal cell death occurring primarily in the cortex and striatum (Vonsattel et al., 1985). The disorder is caused by a CAG/polyglutamine (polyglu) repeat expansion in the first exon of a gene encoding a large ~350 kDa protein of unknown function and designated

Thus, a large body of data has accumulated that describes aspects of the pathology of the above-discussed diseases. However, the actual mechanisms leading to the onset of the various disease states are still unknown. Although a variety of hypotheses have been formulated in the art, it is equally unknown how the amyloid or aggregate formation is triggered or caused within affected cells or tissues. Without a detailed knowledge of the formation of said aggregates, the development of a suitable pharmaceutical composition for treating such diseases appears rather difficult. The technical problem underlying the present invention was therefore to provide means and methods suitable for the eventual elucidation of the etiology of these diseases and the development of appropriate medicines.

been ✓

The above technical problem is solved by the embodiment characterized in the claims.

Accordingly, the present invention relates to a composition comprising

- (a) a nucleic acid molecule encoding a fusion protein comprising
- (aa) a (poly)peptide that enhances solubility and/or prevents aggregation of said fusion protein; and
- (ab) an amyloidogenic (poly)peptide that has the ability to self-assemble into amyloid-like fibrils or protein aggregates;
- (b) a vector containing the nucleic acid molecule of (a);
- (c) a host transformed with the vector of (b);
- (d) a fusion protein encoded by the nucleic acid of (a) or a functional derivative thereof; and/or
- (e) an antibody specific for the fusion protein of (d).

As used herein, the term "(poly)peptide" relates to a polypeptide or a peptide depending on the length of the amino acid string. Said (poly)peptide has the ability to enhance solubility of a fusion partner in said fusion protein and thus of the fusion protein itself. Additionally, or alternatively, said (poly)peptide prevents the aggregation of the fusion partner and thus of the fusion protein. Said (poly)peptide is combined within said fusion protein with an amyloidogenic (poly)peptide having the above recited features. The connection of both (poly)peptides may be via a linker or by a direct attachment. It is preferred that either the linker or either (poly)peptide comprises a cleavable site. Said cleavable site should render both (poly)peptides essentially intact. Alternatively, said fusion protein may comprise a number of cleavage sites. Upon cleavage, which may be exhaustive or under limiting conditions, the amyloidogenic (poly)peptide should, when used for the purposes of

The figures show:

Figure 1

SDS-PAGE Analysis of Purified GST and GST-HD Fusion Proteins.

(a) Aliquots (15 ml) of eluates from the glutathione agarose column were subjected to 12.5 % SDS-PAGE and analyzed by staining with Coomassie blue R. Lanes 1-6 contain GST, GST-HD20, -HD30, -HD83 and -HD122, respectively; lane M contains molecular mass standards. (b) Proteins were transferred to nitrocellulose and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis.

Figure 2

Structure of GST-HD fusion proteins.

The amino acid sequence corresponding to exon 1 of huntingtin is boxed. Arrows labeled Xa and T indicate cleavage sites for factor Xa and trypsin, respectively.

Figure 3

Site-Specific Proteolysis of GST-HD Fusion Proteins with Trypsin and Factor Xa.

Tryptic digestions were performed at 37°C for 3 (a) or 16 h (b). Native proteins and their cleavage products were subjected to 12.5% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis. (c) Purified fusion proteins and their factor Xa and trypsin cleavage products were analyzed using the filter retardation assay. The proteins retained by the cellulose acetate and nitrocellulose membranes were detected by incubation with the anti-HD1 antibody.

Figure 4

Electron Micrographs of Native GST-HD Fusion Proteins and their Factor Xa and Trypsin Cleavage Products.

Purified GST fusion proteins were protease treated, negatively stained with uranyl acetate and viewed by electron microscopy. The undigested GST-HD51 molecules appear as a homogeneous population of small, round particles (a).

and 122 (-HD122) glutamines, respectively, were used for protein purification. For plasmid construction lambda phage from stock 9197₄ (Sathasivam et al., 1997) were plated to give single plaques which were innoculated into 400 ml cultures of *E. coli* XL1-Blue MRF' (Stratagene) for DNA preparation. The DNA sequence encoding the N-terminal portion of huntingtin (exon 1), including the CAG repeats, was amplified by PCR using the following pair of primers: ES 25 (TGGGATCCGATGGCGACCCTGGAAAAGCTGATGAAGG) (Seq. ID No. 1) corresponding to nt315-343 of the HD gene (HDCRG, 1993) and containing a BamHI site (underlined) and ES 26 (GGAGTCGACTACGGTCGGTGCAGC GCTCCTCAGC) (Seq. ID No. 2) corresponding to nt516-588 and containing a Sall site (underlined). Conditions for PCR were as described (Mangiarini et al. 1996). Due to instability of the CAG repeat during propagation in *E. coli*, DNA preparations from individual plaques yielded different sized PCR products. Fragments of ~320, 360, 480, and 590 bp were gel-purified, digested with BamHI and Sall and inserted into the BamHI-Sall site of the expression vector pGEX-5X1 (Pharmacia) yielding pCAG30, pCAG51, pCAG83 and pCAG122, respectively. PCAG20, containing 20 repeats of CAG within the cloned HD exon 1 sequence, was similarly constructed from a phage genomic clone derived from a normal allele. All constructs were verified by sequencing. After induction with IPTG, the resulting proteins were purified under native conditions by affinity chromatography on glutathione agarose. Thus, *E. coli* SCS1 (Stratagene) carrying the pGEX expression plasmid of interest was grown to an OD_{600nm} of 0.6 and induced with IPTG (1 mM) for 3.5 h as described in the manufacturer's protocol (Pharmacia). Cultures (200 ml) of induced bacteria were centrifuged at 4000 g for 20 min, and the resulting pellets were stored at -80°C. Cells were thawed on ice and resuspended in 5 ml of lysis buffer (50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.5 mg/ml lysozyme. After 45 min. at 0°C, cells were sonicated with two 30 sec-bursts. Octyl- β -D-glucopyranoside was then added to a final concentration of 0.1% and the resulting lysate was clarified by centrifugation at 30,000 g for 30 min. at 4°C. Cleared lysates were incubated for 1 h at 4°C with 500 μ l of a 1:1 slurry of glutathione-agarose beads (Sigma) that had been washed times and resuspended in lysis buffer. The beads were poured into a small column and washed extensively with lysis buffer containing 0.1% octyl- β -D-glucopyranoside. The bound fusion protein was eluted with 2 ml of 15 mM glutathione (reduced) in lysis buffer. Typical yields were 0.5-1 mg of purified GST-HD20, -HD30 and -HD51 proteins per 200 ml of bacterial culture; yields of GST-HD83 and -HD122 were much lower, less than 10% of that obtained with the shorter fusion proteins. Protein was determined by the Bio-Rad dye binding assay using bovine serum albumin as standard. SDS-PAGE of the purified GST-HD20, -HD30, -HD51, -HD83 and -HD122

(Lancer) and collected in serial order in 0.1 M phosphate buffer. After being osmicated (30 min in 1% OsO₄ in 0.1 M phosphate buffer) the sections were stained for 15 min in 0.1 % uranyl acetate in sodium acetate buffer at 4 °C, dehydrated in ethanols, cleared in propylene oxide and embedded in Araldite between two sheets of Melanex (ICI). Semi thin (1 µm) sections were cut with glass knives and stained with toluidine blue adjacent to thin sections cut with a diamond knife on a Reichert Ultracut ultramicrotome. The sections were collected on mesh grids coated with a thin formvar film, counterstained with lead citrate and viewed in a Jeol 1010 electron microscope. An electron micrograph of a NII from a 17 month old R6/5 homozygous mouse is shown in Fig. 6c. This NII (large arrow) contains high molecular weight fibrous structures which were clearly differentiated from the surrounding chromatin. The filaments were randomly oriented, 5-10 nm in diameter and often measured up to 250 nm in length (small arrows). These structures differ from those previously reported in the NIIs seen in hemizygous R6/2 mice which were far more granular in composition, with individual filamentous structures being more difficult to distinguish (Davies et al., 1997). R6/2 mice exhibit an earlier age of onset with a more rapid progression of the phenotype and do not survive beyond 13 weeks (Mangiarini et al., 1996). It is possible that the filamentous structures do not have time to form in the R6/2 mice.

Example 5:

Construction of further plasmids, purification of corresponding GST fusion proteins and proteolytic cleavage of GST fusion proteins

In a second set of experiments, a further number of plasmids was constructed. Standard protocols for DNA manipulations were followed (J. Sambrook, E.F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989). *IT-15* cDNA sequences (HDCRG, *Cell* 72, 971 (1993)) encoding the N-terminal portion of huntingtin, including the CAG repeats, were amplified by PCR using the oligonucleotides ES25 (5'-TGGGATCCGCATGGCG ACCCTGGAAAAGCTGATGAAGG-3') and ES27 (3'-CTCCTCGAGCGGCGG TGGCGGCTGTTGCTGCTGCTG-5') as primers and the plasmids pCAG20 and pCAG51 as template (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* 90, 549 (1997)). Conditions for PCR were as described (L. Mangiarini, K. Sathasivam, M. Seller, B. Cozens, A. Harper, C. Hetherington, M. Lawton, Y. Trottier, H. Lehrach, S.W. Davies, and G.P. Bates, *Cell* 87, 493 (1996)). The resulting cDNA fragments were gel purified, digested with *Bam* HI and *Xho* I and were inserted into the

Bam HI-*Xho* I site of the expression vector pGEX-5X-1 (Pharmacia), yielding pCAG20DP and pCAG51DP, respectively. The plasmids pCAG20DP-Bio and pCAG51DP-Bio were generated by subcloning the PCR fragments obtained from the plasmids pCAG20 and pCAG51 into pGEX-5X-1-Bio. pGEX-5X-1-Bio was created by ligation of the oligonucleotides BIO1 (5'-CGCTCGAGGGTATCTTCGAGGCC
AGAAGATCGAGTGGCGATCACCATGAG-3') and BIO2 (5'-GGCCGCTCATGGTG
ATCGCCACTCGATCTTCTGGGCCTCGAAGATAACCCTCGAG-3'), after annealing and digestion with *Xho* I, into the *Xho* I-*Not* I site of pGEX-5X-1. The plasmids with the *IT-15* cDNA inserts were sequenced to confirm that no errors had been introduced by PCR. The construction of plasmids pTL1-CAG20, pTL1-CAG51 and pTL1-CAG93 for the expression of huntingtin exon 1 proteins containing 20, 51 and 93 glutamines in mammalian cells has been described (A. Sittler, S. Wälter, N. Wedemeyer, R. Hasenbank, E. Scherzinger, G. P. Bates, H. Lehrach, and E. E. Wanker, *Mol. Cell*, submitted).

The amino acid sequence of the GST-HD fusion proteins encoded by the *E. coli* expression plasmids pCAG20DP, pCAG51DP, pCAG20DP-Bio and pCAG51DP-Bio is shown in Fig. 8. The plasmids pCAG20DP and pCAG51DP encode fusion proteins of glutathione S-transferase (GST) and the N-terminal portion of huntingtin containing 20 (GST-HD20DP) and 51 (-HD51DP) polyglutamines, respectively. In these proteins the proline-rich region located immediately downstream of the glutamine repeat was deleted (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* 90, 549 (1997)). The fusion proteins GST-HD20DPBio and -HD51DPBio are identical to GST-HD20DP and -HD51DP, except for the presence of a biotinylation site (P. J. Schatz, *Biotechnology* 11, 1138 (1993)) at their C-termini.

In the experiments described herein, *E. coli* DH10B (BRL) was used for plasmid construction and *E. coli* SCS1 (Stratagene) was used for the expression of GST-HD fusion proteins. Transformation of *E. coli* with plasmids and ligation mixtures was performed by electroporation using a Bio-Rad Gene Pulser (Richmond, CA). Transformed cells were spread on LB plates supplemented with appropriate antibiotics (J. Sambrook, E.F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989). For expression of GST fusion proteins, cells were grown in liquid TY medium (5 g NaCl, 5 g yeast extract, and 10 g tryptone per liter) buffered with 20 mM MOPS/KOH (pH 7.9) and supplemented with glucose (0.2%), thiamine (20 µg/ml), ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

S. Igarashi, R. Koide, T. Shimohata, M. Yamada, Y. Hayashi, H. Takano, H. Date, M. Oayke, T. Sato, A. Sato, S. Egawa, T. Ikeuchi, H. Tanaka, R. Nakano, K. Tanaka, I. Hozumi, T. Inuzuka, H. Takahashi, and S. Tsuji, *Nature Genetics* 18, 11 (1998).

P. J. Skinner, B. T. Koshy, C. J. Cummings, I. A. Klement, K. Helin, A. Servadio, H. Y. Zoghbi, and H. T. Orr, *Nature* 389, 971 (1997).

A. Matilla, B. T. Koshy, C. J. Cummings, T. Isobe, H. T. Orr, and H. Y. Zoghbi, *Nature* 389, 974 (1997).

H. L. Paulson, M. K. Perez, Y. Trottier, J. Q. Trojanowski, S. H. Subramony, S. S. Das, P. Vig, J.-L. Mandel, K. H. Fischbeck, and R. N. Pittman, *Neuron* 19, 333 (1997).

M. Holmberg, C. Duyckaerts, A. Durr, G. Cancel, I. Gourfinkel-An, P. Damier, B. Faucheux, Y. Trottier, E. C. Hirsch, Y. Agid, and A. Brice, *Hum. Mol. Genet.* 7, 913 (1998).